Remarks

The specification was amended to correct typographical errors contained within the original application filing. No new matter has been added. No additional search or examination is necessitated by the amendment, nor is any substantial amount of additional work required on the part of the Patent Office or the Examiner.

Conclusion

The Examiner is invited to contact Applicants' Representatives at the below-listed telephone number, if there are any questions regarding this Preliminary Amendment or if prosecution of this application may be assisted thereby.

CERTIFICATE UNDER 37 C.F.R. 1.8:

The undersigned hereby certifies that this paper is being deposited in the United States Postal Service, as first class mail, in an envelope addressed to: Assistant Commissioner for Patents, Attn: Box Missing Parts, Washington, D.C. 20231, on this 18 day of July, 2001.

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July 18, 2001
Date

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APPENDIX A - SPECIFICATION/CLAIM AMENDMENTS INCLUDING NOTATIONS TO INDICATE CHANGES MADE
Serial No.: 09/747,420
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Amendments to the following are indicated by underlining what has been added. Additionally, all amendments have been shaded.

In the Specification

The paragraph beginning at page 4, lines 15-18, has been amended as follows:

Figure 1 is an illustration of the chemical structures of inhibitors used in co-crystallization experiments. Figure 1(A) is the synthetic peptide Ser-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe-Arg-Gly-Gly-Cys (where Sta = statine), SEQ ID NO:3. Figure 1(B) is another inhibitor.

The paragraph beginning at page 34, line 14, through page 35, line 4, has been amended as follows:

The expression plasmid = pcDNA3.1/myc/his (neomycin) (Invitrogen) contains beta secretase extending from Met [-21] to Ser [432] with a myc tag followed by a hexahistidine tag [EQKLISEEDLNMHTEHHHHHH*], SEQ ID NO:2, at the C-terminus. Following transfection in HEK293 cells, stable cells were selected using 0.8 mg/ml G418. A stable clone of transfected HEK293 cells that secretes human beta-secretase was expanded in static, monolayer cell culture. Confluent cultures were detached by shaking and a plurality of plastic, 225 cm^2 T-flasks were each inoculated with a suspension of 1-5 x 106 cells in 100 ml of High-Glucose Dulbecco's Modified Eagle medium that was supplemented with 5% fetal bovine serum and 500 micrograms/ml G418. These cell cultures were incubated in a humidified. 37°C incubator gassed with 95% air and 5% $C0_2$. Once the cells reached confluence the growth medium in each flask was removed and replaced with 100 ml fresh medium. The conditioned, culture medium supernatant was harvested aseptically and replaced by fresh medium every 48-72 hours. The harvested medium was pooled, centrifuged at 1000 x g to remove cell debris, and was stored in plastic bottles at 4°C. Cell monolayers were maintained in semi-continuous culture for several weeks until the cells either began to die or to detach from the culture flasks. The cells were then resuspended and used to inoculate a fresh set of production flasks.

The paragraph beginning at page 35, line 15, through page 36, line 7, has been amended as follows:

The 40-80% ammonium sulfate pellet was dissolved in 25 mM Tris-HCI (8.5)10.5 M NaCIIIO mM imidazole (1/10 the original volume) and applied to a 12.5 ml column containing Ni^{+}-NTA Fast Flow resin previously equilibrated in the same buffer. Following sample application, the column was washed with 10 column volumes of loading buffer and then eluted with 25 mM Tris-HC1 (8.5)/0.5 M NaCl/50 mM imidazole. The material eluting in 50 mM imidazole was pooled, concentrated approximately 10-fold using a YM 30 membrane (30,000 MWCO), and then dialyzed against 10 mM HEPES-Na (8.0) using 50,000 molecular weight cutoff tubing. For affinity purification, the synthetic peptide Ser-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe-Arg-Gly-Gly-Cys (where Sta = statine, PNU-292593E), SEQ ID NO:3, was synthesized and coupled to sulfolink resin (Pierce Chemical Company) as recommended by the manufacture. The dialyzed material from above was adjusted to 0.1 M NaOAc (4.5) by addition of 1/10 volume of 1.0 M NaOAc (4.5) and immediately applied to the PNU-292593E/sulfolink column (6 ml containing 1.0 mg PNU-292593/ml of resin) that had been previously equilibrated in 25 mM NaOAc (4.5). Following sample application, the column was washed with 10 column volumes of 25 mM NaOAc (4.5) and then eluted with 50 mM $NaBO_3$ (8.5). N-terminal sequence analysis of the affinity purified material revealed an equimolar mixture of pro- and processed human beta-secretase beginning at Thr^{1} and Glu^{25} respectively. The final protein concentration was determined by amino acid analysis assuming a 60 kDa glycoprotein.

The paragraph beginning at page 36, lines 8, through page 37, line 3, has been amended as follows:

Production of Recombinant Human β-Secretase in Insect sf9 Cells and CHO-K1 Cells. The coding sequence was engineered to delete the terminal transmembrane and cytoplasmic domain and introduce a C-terminal hexahistidine tag using the polymerase chain reaction. The 5' sense oligonucleotide primer

[CGCTTTGGATCCGTGGACAACCTGAGGGGCAA], SEQ ID NO:4, was designed to incorporate a BamHI site for ease in subcloning and Kozak consensus sequence around the initiator methionine for optimal translation initiation. The 3' antisense primer [CGCTTTGGTACCCTATGACTCATCTGTCTGTGGAATGTTG], SEQ ID NO:5, incorporated a hexahistidine tag and translation termination codon just upstream of the predicted transmembrane domain (Ser^{432}) and a NotI restriction site for cloning. The PCR was performed on the plasmid template pcDNA3.1hygroAsp2R for 15 cycles [94°C, 30 sec., 65°C, 30 sec., 72°C, 30 sec] using Pwo I polymerase (Roche Biochemicals, Indianapolis, IN) as outlined by the manufacturer. The PCR product was digested to completion with *Bam*HI and *Not*I and ligated into the *BamH*I and *Not*I sites of the baculovirus transfer vector pVL1393 (PharMingen, San Diego, CA). A portion of the ligation was used to transform competent E. coli DH5α cells and recombinant clones were selected on ampicillin. Individual clones containing the proper cDNA inserts were identified by PCR. Plasmid DNA from clone (pVL1393/Hu_Asp-2LΔTM$(His)_6$) was prepared by alkaline lysis and banding in CsCl. The integrity of the insert was confirmed by complete DNA sequencing. For CHO-K1 cell expression, plasmid pVL1393/Hu_Asp-2LΔTM$(His)_6$ was digested with *BamH*I and *Not*I and the resulting fragment subcloned into the mammalian expression vector pcDNA3.1(hygro) as described above to yield pcDNA3.1(hygro)/ Hu_Asp-2LΔTM$(His)_6$).

The paragraph beginning at page 38, line 16, through page 39, line 9, has been amended as follows:

The 40-80% ammonium sulfate pellet was dissolved in 25 mM Tris-HCl (8.5)/0.5 M NaCl/10 mM imidazole (1/10 the original volume) and applied to a 12.5 ml column containing Ni^{+}-NTA Fast Flow resin previously equilibrated in the same buffer. Following sample application, the column was washed with 10 column volumes of loading buffer and then eluted with 25 mM Tris-HCl (8.5)/0.5 M NaCl/50 mM imidazole. The material eluting in 50 mM imidazole was pooled, concentrated approximately 10-fold using a YM 30 membrane

(30,000 MWCO), and then dialyzed against 10 mM HEPES-Na (8.0) using 50,000 molecular weight cutoff tubing. For affinity purification, the synthetic peptide Ser-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe-Arg-Gly-Gly-Cys (where Sta = statine, PNU-292593E), SEQ ID NO:3, was synthesized and coupled to sulfolink resin (Pierce Chemical Company) as recommended by the manufacture. The dialyzed material from above was adjusted to 0.1 M NaOAc (4.5) by addition of 1/10 volume of 1.0 M NaOAc (4.5) and immediately applied to the PNU-292593E/sulfolink column (6 ml containing 1.0 mg PNU-292593/ml of resin) that had been previously equilibrated in 25 mM NaOAc (4.5). Following sample application, the column was washed with 10 column volumes of 25 mM NaOAc (4.5) and then eluted with 50 mM $NaBO_3$ (8.5). N-terminal sequence analysis of the affinity purified material revealed an equimolar mixture of pro- and processed human β-secretase beginning at Thr^1 and Glu^{25}, respectively. The final protein concentration was determined by amino acid analysis assuming a 52 kDa glycoprotein for insect cells and a 60 kDa glycoprotein for CHO cells, respectively.

The paragraph beginning at page 39, line 25, through page 40, line 17, has been amended as follows:

The 40-80% ammonium sulfate pellet was dissolved in 25 mM Tris-HCl (8.5)/0.5 M NaCl/10 mM imidazole (1/10 the original volume) and applied to a 12.5 ml column containing Ni^+-NTA Fast Flow resin previously equilibrated in the same buffer. Following sample application, the column was washed with 10 column volumes of loading buffer and then eluted with 25 mM Tris-HCl (8.5)/0.5 M NaCl/50 mM imidazole. The material eluting in 50 mM imidazole was pooled, concentrated approximately 10-fold using a YM 30 membrane (30,000 MWCO), and then dialyzed against 10 mM HEPES-Na (8.0) using 50,000 molecular weight cutoff tubing. For affinity purification, the synthetic peptide Ser-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe-Arg-Gly-Gly-Cys (where Sta = statine, inhibitor shown in Figure 1A), SEQ ID NO:3, was synthesized and coupled to sulfolink resin (Pierce Chemical Company) as recommended by the manufacture. The dialyzed material from above was adjusted to 0.1 M

NaOAc (4.5) by addition of 1/10 volume of 1.0 M NaOAc (4.5) and immediately applied to the inhibitor shown in Figure 1A/sulfolink column (6 ml containing 1.0 mg of the inhibitor shown in Figure 1A/ml of resin) that had been previously equilibrated in 25 mM NaOAc (4.5). Following sample application, the column was washed with 10 column volumes of 25 mM NaOAc (4.5) and then eluted with 50 mM $NaBO_3$ (8.5). N-terminal sequence analysis of the affinity purified material revealed an equimolar mixture of pro- and processed human β-secretase beginning at Thr^1 and Glu^{25}, respectively. The final protein concentration was determined by amino acid analysis assuming a 60 kDa glycoprotein.